Genomics to Genetics: EOMES is a novel partner of MITF-PU.1 regulated transcriptome in osteoclast differentiation

Research Thesis

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by

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Vitae

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Abstract

Bone remodeling is a continuous process regulated by bone-forming cells, osteoblasts, and bone-degrading cells, osteoclasts. Osteoclasts are derived from myeloid precursors. Increased differentiation and activation of osteoclasts offsets the balance of bone remodeling, causing the painful bone degradation associated with diseases such as osteoporosis, rheumatoid arthritis, and bone metastasis in cancer patients.

Osteoclast differentiation is brought about by an orchestrated transcription program in response to micro-environmental signaling. The full extent of this transcription factor network is not understood. Two transcription factors, MITF and PU.1, have been found to jointly regulate the transcription of many genes critical to the differentiation of myeloid precursor cells to osteoclasts. Through the use of chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-Seq), we identified the genomic areas in developing osteoclasts enriched for MITF/PU.1 binding. Motif analysis revealed that 38% of these areas also included binding sites for the T-box transcription factor EOMES. Included in these sites were members of the complex transcription factor network governing osteoclast differentiation, such as NFATC1 and C-FOS.

To evaluate the physiological significance of transcription factor EOMES, we developed a myeloid specific knockout mouse model of EOMES. These mice had an osteopetrotic phenotype caused by reduced osteoclast differentiation and activity. These results were explained by a decrease in the expression of genes necessary for osteoclast differentiation, including *Nfatc1*. Therefore, we concluded that EOMES is another crucial transcription factor in early osteoclast differentiation. Understanding the intricate mechanisms controlling osteoclast differentiation is important for creating targets for therapeutic treatments of bone-related ailments.

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Acknowledgements

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I. Introduction

a. Bone Structure and Remodeling

Bone is a living, growing tissue that provides structure and support to the human body. It is made up two layers: the hard outer layer, the cortical bone, and the spongy inner layer, the trabecular bone. These layers consist of organic collagen, a protein that provides the framework, and minerals that give the bone strength, such as hydroxyapatite (1). Not only are bones necessary for locomotion, they also serve as a protective layer for vital organs. As one of the first-lines of defense against outside stresses, bones are constantly accumulating damage.

To maintain the integrity of the bone, a mechanism is in place to remove the damaged bone and replace it with new, healthy bone: bone remodeling. Bone remodeling is a continuous, lifelong process that responds to the persistent buildup of damage, and consists of two phases: resorption and ossification. Resorption involves osteoclasts, cells responsible for the degradation of the damaged bone. Osteoclasts originate from the hematopoietic stem cells, found in the bone marrow (2). Following resorption, osteoblasts, cells derived from the mesenchyme cell lineage, build new bone. The perfect balance of these two cells maintains the strength and mass of the bone.

b. Bone-related Ailments

Imbalance of the osteoblast-osteoclast relationship is responsible for an assortment of bone-related ailments. Each year, approximately 1.5 million individuals suffer from bone fractures attributed to bone-related ailments (3). In the case of under activity of osteoclasts, the normal activity of osteoblasts trumps, causing the bones to become very dense. This condition is known as osteopetrosis. Osteopetrosis is a rare, autosomal recessive disorder. Symptoms of this

condition include fractures, low blood-cell production, and loss of cranial-nerve function. This could lead to blindness, deafness, and facial nerve paralysis (4).

More common, however is the hyperactivity of osteoclast (5). The most prevalent disease associated with hyperactivity of osteoclast is osteoporosis, a disease that leaves bones susceptible to damage due to the loss of bone mass and strength. Even mild stress to the bones can lead to a fracture. Osteoporosis causes shortened height, curvature to the back, reduced mobility, and pain in the bones (3). The disease is most common in post-menopausal women; estrogen decreases osteoclast production, and post-menopausal women experience a drastic drop in estrogen levels (6). Although it is hard to diagnose before a fracture occurs, it is estimated that roughly 200 million women worldwide suffer from osteoporosis (3).

Hyperactivity of osteoclasts is also present in rheumatoid arthritis. Rheumatoid arthritis is characterized by severe joint pain and swelling, stiffness and fatigue in the individual (7). It is an autoimmune disease, where immune cells become deregulated and attack the healthy cartilage of the joints (8). The bone destruction associated with rheumatoid arthritis is due to the overproduction and activation of osteoclasts. Rheumatoid arthritis affects approximately 1.3 million Americans, seventy-five percent of which are female (5).

Along with these bone-related ailments, hyperactivity of osteoclasts plays a role in some cancers. Tumors can produce or induce signals that stimulate the production of osteoclasts (9). These degrade the bone, and supply cancer cells with a pathway for bone metastasis. Bone metastasis is very painful and can lead to bone fractures, adding to the long list of sufferings experienced by cancer patients.

c. Osteoclast Function and Differentiation

Osteoclasts' function is carried out by three membrane domains: the sealing zone, the ruffled border, and the functional secretory domain. The sealing zone is the area of the cell that attaches to the bone matrix. The ruffled border provides hydrochloric acid and proteases to the resorption lacuna, an area between the border and the bone (10). Hydrochloric acid functions to degrade the minerals, such as crystalline hydroxyapatite, and the proteases degrade the collagen matrix of the bone. The resulting fragments are endocytosed, processed, and secreted by the functional secretory domain (10). Osteoclasts function at both the trabecular level and the cortical bone level, leading to an overall decrease in the volume of the bone.

Active, multinucleated osteoclasts are differentiated from myeloid stem cells via signals from two cytokines: macrophage colony-stimulating factor-1 (CSF-1) and receptor activator of nuclear factor (NF)-kB ligand (RANKL). CSF-1 is produced by osteoblasts and stromal cells, and plays a crucial role in the proliferation and survival of osteoclast precursors (11). After binding to the c-Fms receptor, a receptor of the tyrosine kinase family, the signal is relayed by the ERK1/2 and PI3K/Akt pathway. This induces the expression of genes crucial to promote the survival and differentiation of osteoclast precursors. Among these is the gene encoding the RANK receptor. The expression and transport of these receptors to the cell membrane prepares the osteoclast precursor for the subsequent signal (11). RANKL is also released by osteoblasts and stromal cells, and binds to the RANK receptors presented on the osteoclast precursors (5). This receptor-ligand complex recruits adaptor molecules to the cytoplasmic domain of RANK, which initiate the downstream regulation of osteoclast effector genes, effectively changing the physiology of the cell and producing multinucleated, active osteoclasts (5).

d. Osteoclast Transcription Factor Network

While these extracellular signals are necessary for the differentiation process, their cues must be propagated by the intracellular regulatory systems they activate. Signals by CSF-1 and RANKL lead to downstream activation of a complex transcription factors network. This intricate network contains factors that can have multiple functions in the differentiation process, such as regulate other transcription factors in the network, increase expression of osteoclast effector genes, and inhibit the expression of genes that would stall differentiation. Important members of the transcription factor network include a member of activator protein 1 (C-FOS), nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), E26 oncogene homolog family transcription factor (PU.1), and micropthalmia-associated transcription factor (MITF) (5,12-14).

These transcription factors regulate and maintain the expression of osteoclast effector genes that change the physiology of the myeloid precursor to active osteoclast. Some of the important genes upregulated following the activation of the network include tartrate-resistant acid-phosphatase (TRAP) gene (*Acp5*), which has important enzymatic function in resorption, cathepsin K (*Ctsk*), responsible for the degradation of collagen, osteoclast-associated receptor (*Oscar*), and calcitonin receptor (*Calcr*) (15-18), a receptor which binds calcitonin, a hormone important for calcium homeostasis.

The transcription factor network has captured the interest of many researchers. Members of the network play large roles in many other cell types, but in osteoclast differentiation, the interactions of all of these unique transcription factors are necessary to elicit an osteoclast-specific response.

e. CFOS -/- mice and NFATC1

Along with its function in osteoclast differentiation, C-FOS is a marker of activated neurons, and its overexpression has been found in invasive mammary epithelial cells. C-FOS is a crucial member of osteoclast differentiation, as it is necessary for the expression of NFATC1 (19). NFATC1 functions late in the transcription factor network. It is only activated following RANKL signaling, and plays a role in the maintenance of the osteoclast-effector genes. Apart from its role in osteoclast development, NFATC1 participates in immune responses.

Mice lacking C-FOS have an osteopetrotic phenotype and reduction in *Nfatc1* expression. When NFATC1 is reintroduced into these mice, it restores the normal osteoclast production (19). C-FOS and NFATC1 have roles in different cell types, but C-FOS regulation of NFATC1 is necessary for osteoclast differentiation. The function of NFATC1 is vital for sustaining the active osteoclast physiology during its function.

f. PU.1 conditional knockout and MITF mutant mouse models

The Ostrowski-Sharma laboratory has been especially interested in the relationship of MITF and PU.1, two functionally distinct transcription factors with an early, specific role in osteoclast differentiation.

PU.1 is present and active in all hematopoietic cells, making its expression necessary for the formation of macrophages, neutrophils, B-cells, and T-cells, in addition to osteoclasts (20). It contains an ETS binding domain of eighty-one amino acids, which binds to a highly conserved GGAA binding site on DNA. While PU.1 does not affect the commitment of hematopoietic cells to particular lineages, it is necessary for the process of differentiation to these lineages (20).

MITF is a basic helix-loop-helix zipper transcription factor; its binding domain recognizes an E-box motif. After binding to this DNA site, it can control the regulation of lineage-specific genes by association with co-activators or co-repressors (21). Apart from its role in osteoclast differentiation, MITF functions in the development of mast cells, melanocytes, and optic pigment cells (21). In these cells, the role of MITF is extensive; it participates in the regulation of cell specification, proliferation, and differentiation.

Because of its role in all hematopoietic cells, complete knockout of PU.1 is embryonic lethal. To circumvent this obstacle, a myeloid-specific, inducible knockout of PU.1 was bred in the Ostrowski-Sharma laboratory. Following the conditional knockout of PU.1, the mice showed

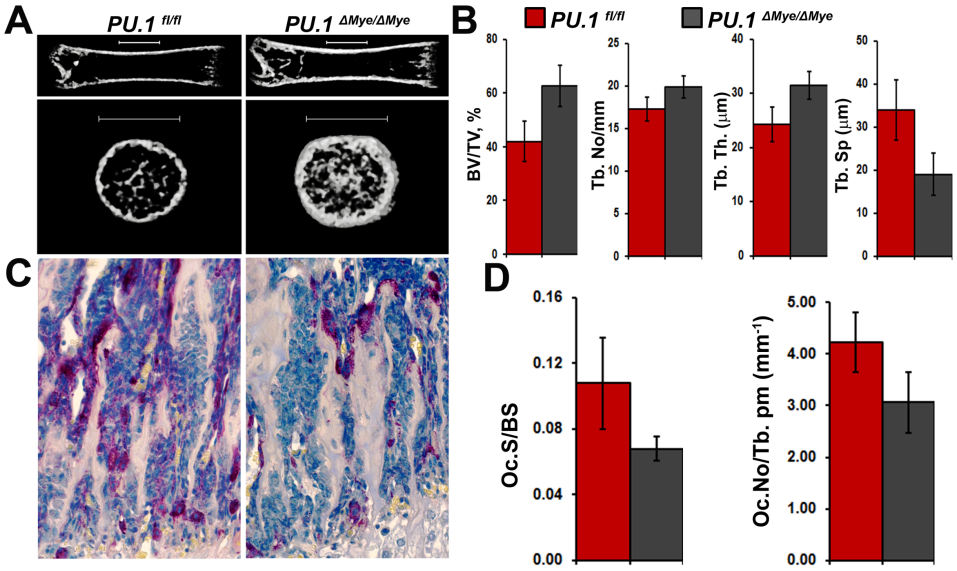

Figure 1: A. Micro CT image demonstrating osteopetrosis in PU.1 cKO mice B. Quantification of the percentage of bone volume to total volume. C. The femur in Figure *A* stained for tartrate resistant acid phosphatase (TRAP), a common osteoclast marker of osteoclast, showing a decrease in functional osteoclast production in PU.1 cKO mice. D. Quantification of osteoclast surface area to total bone surface area of PU.1 cKO mice compared to controls.

an osteopetrotic phenotype (unpublished: Carey, H. and Hildreth, Figure 1A, B). Rather than a deletion of MITF, MITF was mutated in the basic domain of the gene, making it incapable of binding to DNA. This model showed also showed an osteopetrotic phenotype (22).

To explain the osteopetrotic phenotypes of these mice, the femurs were stained for the TRAP protein to quantify active osteoclast present on the bone. In the mice lacking PU.1 and functional MITF, there was decreased osteoclast present on bone surface and on the perimeter of the trabecular (22) (unpublished: Carey, H. and Hildreth, E., Figure 1C,D).

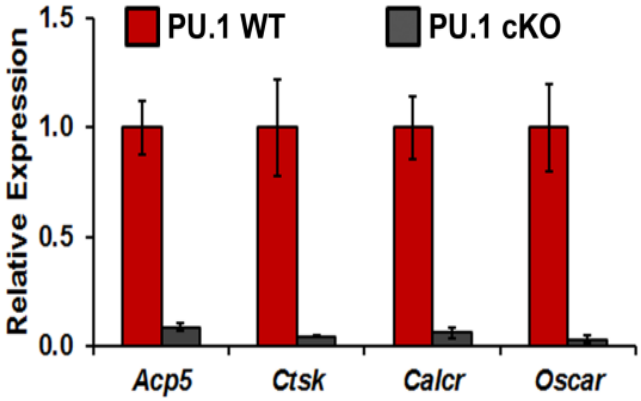

Figure 2: qPCR Gene Expression analysis shows a dramatic decrease of osteoclast effector gene expression in PU.1 knockout mice.

The overall cause of decreased osteoclast production was decreased expression of osteoclast effector genes in mice lacking PU.1 and functional MITF (Figure 2). These results were found using primers of osteoclast-specific genes for quantitative real time PCR (qRT-PCR) of RNA isolated from mouse bone marrow-derived macrophages (BMMs) treated with CSF-1 and RANKL.

g. MITF and PU.1 Co-regulation

While the individual regulation of MITF and PU.1 is important in osteoclast differentiation, what has captured the attention of the Ostrowski-Sharma lab is the discovery that the two unique transcription factors physically interact to regulate many osteoclast-specific genes. Upon signaling from CSF-1 in bone marrow precursors, MITF is recruited to the promoter sites of osteoclast effector genes, stimulating the co-recruitment of PU.1 (23). After the combined signaling of RANKL, MITF is activated, and PU.1 and MITF jointly control the expression of the genes.

In one study of MITF/PU.1 co-regulation, focused on *Acp5,* the gene encoding TRAP, MITF and PU.1 binding sites were found to be roughly 10bp apart in the gene (15). With the expression of only PU.1 or MITF in transfection assays, the activation of the *Acp5* promoter increased 4-5 fold following RANKL signaling. But, with combined expression of MITF and PU.1, the activation increased 20-fold (15). Study of *Oscar* expression displayed a 4-5 fold increase in promoter activation with MITF-alone, but up to a 110 fold increase with the function of both transcription factors (17).

Along with genes that have a direct function in changing the physiology of the cell, MITF and PU.1 also regulate the function of important members of the transcription factor network, such as NFATC1 (24). It was shown that MITF/PU.1 binding at the *Ctsk* and *Acp5* promoter stimulated their expression. Expression of these genes was necessary to recruit NFATC1 to its binding site on the *Ctsk* and *Acp5* promoters, where it functions to maintain their expression (24).

h. ChIP-seq of MITF and PU.1

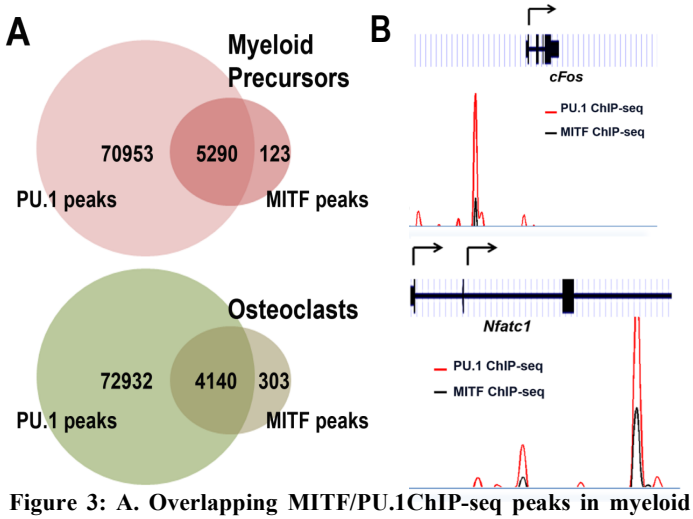

Figure 3: A. Overlapping MITF/PU.1ChIP-seq peaks in myeloid precursors and osteoclast. B. MITF and PU.1 co-bind to promoter regions of *c-Fos* and *Nfatc1*, members of the osteoclast transcription factor network.

The interaction between PU.1 and MITF is one example of the unique regulation process of the osteoclast differentiation: functionally different transcription factors can elicit a very specific response. PU.1 is expressed in all hematopoietic cells; MITF is expressed in a multitude of

cell types. But together, they allow for the expression of osteoclast-specific genes in committed myeloid precursors.

Because of the significance of this complex, the Ostrowski-Sharma laboratory conducted high throughput chromatin-immunoprecipitation sequencing (ChIP-seq) in both myeloid precursors and mature osteoclasts to gain insight into the magnitude of MITF/PU.1 co-regulation. Overlapping the MITF/PU.1 ChIP-seq peaks revealed that the two transcription factors shared over five thousand binding sites in myeloid precursors, and over four thousand binding sites in mature osteoclasts (unpublished: Carey, H. and Sharma, S., Figure 3A). Of these sites of co-binding, over one thousand are involved with genes critical to osteoclast differentiation. Not only did these genes encode for proteins that elicit a direct change in cell type, they also included other transcription factors, such as C-FOS and NFATC1 (unpublished, Carey, H. and Sharma, S., Figure 3B).

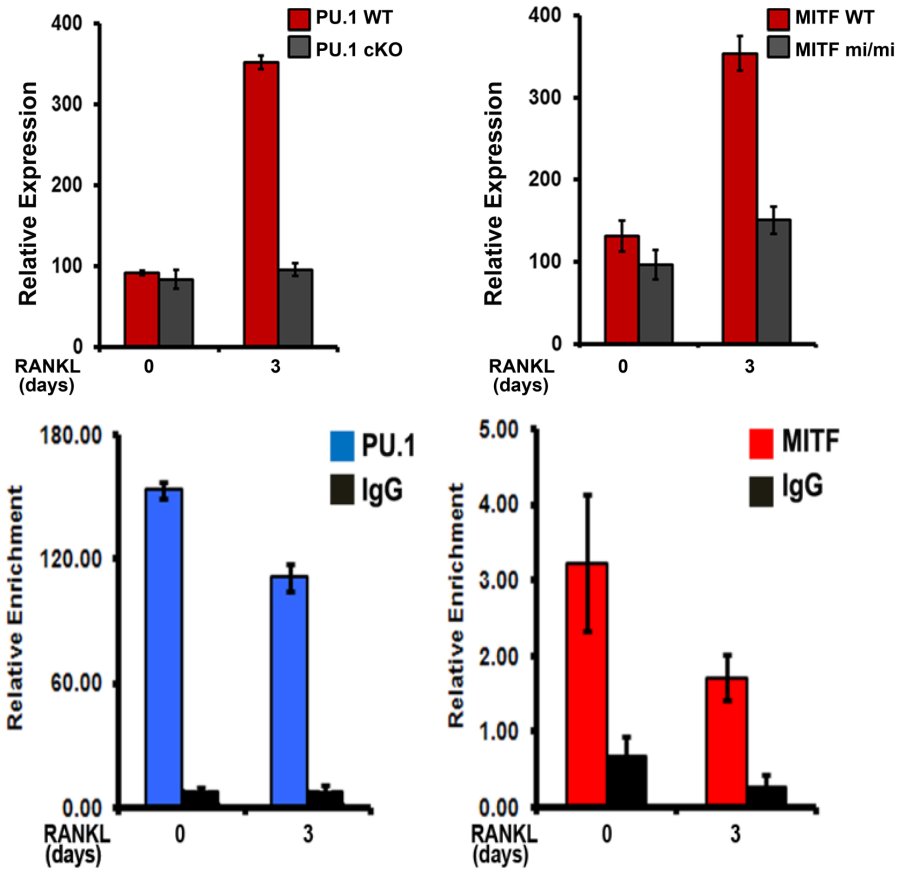

Figure 4: A. ChIP followed by qPCR confirmed MITF/PU.1 binding to peaks of after CSF-1 treatment that persisted following RANKL treatment. B. qPCR of RNA from BMMs treated with cytokines at different time points showed that knockout of PU.1 and mutations of MITF decreased the expression of Nfatc1 following RANKL treatment. PU.1 knockout shows a dramatic decrease in Nfatc1 expression.

Many of these binding sites were confirmed using chromatin-immunoprecipitation followed by RT-PCR, including PU.1 and MITF binding at a site 5kbp from the promoter of *Nfatc1* (Figure 4A). This binding suggests that PU.1 and MITF have important upstream regulatory functions in the transcription factor network.

The transcriptional regulation of *Nfatc1* was confirmed with qRT-PCR analysis using RNA from BMMs treated with CSF-1 and RANKL from wildtype and knockout/mutant PU.1 and MITF mice (Figure 4B). NFACT1 has a central role in maintaining osteoclast function, and has been shown to rescue normal phenotypes in osteopetrotic mice lacking other members of the transcription factor network. These results prove PU.1 and MITF are significant regulators of the transcription factor network.

i. Motif Analysis reveals potential third partner

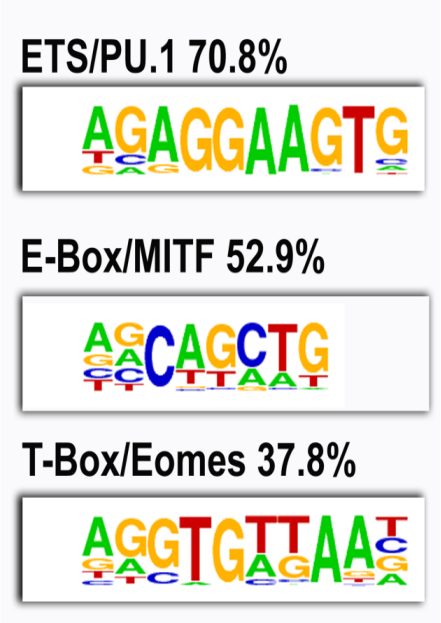

Figure 5: EOMES shares 40% of the sites co-bound by MITF and PU.1, important regulators of osteoclast differentiation.

Because MITF and PU.1 interactions lead to extensive and specific regulation patterns, the Ostrowski-Sharma lab wanted to look into possible co-factors that could assist MITF/PU.1 in their function. To search for the presence of other co-regulators, a motif analysis scored the DNA sites bound by the conserved GGAA binding site of PU.1 and E-box binding site of MITF. This revealed that almost 40% of these sites also contained the sequence for the T-box transcription factor binding site of Eomesodermin (EOMES) (unpublished: Carey, H. and Sharma, S., Figure 5). Included in these

sites bound by all three transcription factors were members of the transcription factor network, including C-FOS and NFATC1.

While EOMES normally functions during embryonic development, assisting in the formation of the mesoderm and central nervous system, this finding suggests that EOMES might also play a crucial role with MITF and PU.1 in the differentiation of osteoclast.

II. Hypothesis

EOMES is a novel transcription factor of osteoclast differentiation, co-regulating osteoclast-specific genes with MITF and PU.1.

Not only did ChIP-seq reveal the extent of the MITF/PU.1 co-regulation, it also exposed a potential third partner involved in the regulation of osteoclast-specific genes. Motif analysis of the peaks bound by MITF/PU.1 found that roughly thirty-eight percent of the shared sites also contained sequences for the binding of the T-box transcription factor, EOMES. EOMES has been previously studied for its role in mesoderm and nervous system formation during embryonic development, and its function in the differentiation of CD8+ T-Cells involved in immune responses (25,26). But, since the EOMES binding sequence is prevalent in MITF/PU.1 binding sites in osteoclasts, it may also have a role in osteoclast differentiation.

Included in these binding regions are members of the intricate transcription factor network of osteoclast differentiation, such as NFATC1 and C-FOS. NFATC1 is necessary to produce and sustain the osteoclast cell type, and has been shown to rescue osteoclast production in mice lacking C-FOS. If MITF, PU.1, and EOMES interact to regulate the expression of *Nfatc1*, it would suggest that these three transcription factors act as early regulators of the

osteoclast transcription factor network, adding to the unique mechanism of osteoclast differentiation.

Specific Aims:

1. Confirm EOMES binding to sites co-bound by MITF and PU.1
2. Analyze bone phenotype and osteoclast production in an EOMES conditional knockout mouse model
3. Establish EOMES regulation in the transcription factor network
4. Study EOMES role in transcription of osteoclast effector genes

III. Methodology

Mice Breeding and conditions: All mice were maintained in the C57BL6/J background. Mice used were housed in a 12-hour light, 12-hour dark environment, with food, water, and care provided within the guidelines of the National Institute of Health and The Ohio State University Animal Care and Use Committee.

Conditional knockdown of EOMES in myeloid cells:

Mice with loxp sites surrounding the *Eomes* allele, $Eomes^{fl/fl}$, were bred with $Eomes^{fl/fl}$ mice with a tamoxifen-inducible Cre recombinase under a c-FMS promoter, $Eomes^{fl/fl}$ *; Fms-ER*^{t}*Cre,* to obtain $Eomes^{fl/fl}$ (control) and $Eomes^{fl/fl}$ *; Fms-ER*^{t}*Cre* (experimental) mice (Figure 6). Neonatal mice with these genotypes

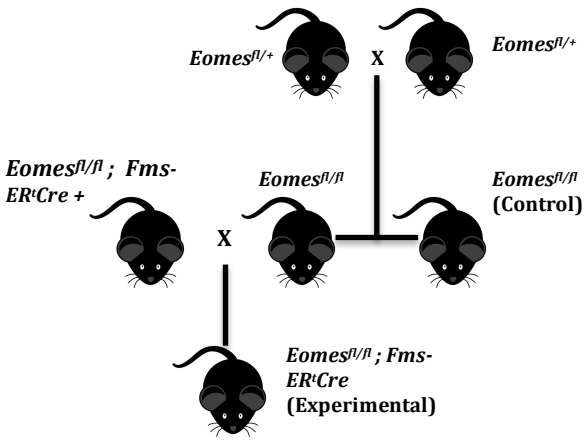

Figure 6: Mice breeding patterns to obtain the experimental and controls mouse models used in *in vivo* studies of osteoclast differentiation.

were injected with tamoxifen (50µg) 3 days after birth. When the mice were 8 days old, they were euthanized. Femurs and tibias were dissected and used for bone marrow flushing or subject to microCT scans to quantify bone defects.

Mouse Genomic DNA Extraction and Preparation: Mouse-tails were clipped, and used as the source of DNA for genotyping. They were digested overnight at 55°C with 0.5mL TE-SDS (50mM Tris-Cl; pH 8.0, 100mM EDTA and 0.5% SDS) and 1µg/mL proteinase K. The DNA was centrifuged and supernatant transferred to a fresh tube containing 0.5mL isopropanol. This was centrifuged, and supernatant discarded. 70% ethanol was added, the mixture centrifuged, and supernatant discarded. 0.5mL Low EDTA was added to the DNA pellet and incubated for 1 hour at 55°C.

Genotyping EOMES mouse models, Polymerase Chain Reaction (PCR): PCR amplication was used for genotyping of the wild-type and knockout mice. Approximately 150ng of extracted mouse DNA was added to a mixture of 1X Thermopol PCR Buffer, 0.2µM deoxy-nucleotide tri phosphates (dNTPs), 0.4µM of both forward and reverse primers for the EOMES allele (Invitrogen) (Table 1), and 2 units of Taq DNA polymerase for a total of 20µL.

Genotype	Forward	Reverse
Eomes (f/f, f/+, wt)	5'-AGATGGAAATTTGGGAAT-3'	5'-GGCTACTACGGCCTGAAACT-3'
FTC 1 (+, -)	5'-TCGAAGCTTGCATGCCTGA-3'	5'-TCTCTGCCCAGAGTCATCCT-3'
FTC 2 (+, -)	5'-TCATTCCAGAACCAGAGC-3'	5'-GATCGTTGGGGAGCC-3'

Table 1: Primers used for genotyping *Eomes*$^{fl/fl}$ and *FmsER*^{i}*Cre* (*FTC*) mice

Genotyping EOMES mouse models, Agarose Gel Electrophoresis*:* Agarose gels for genotyping were prepared by dissolving 2% concentration of agarose (Fisher Scientific) into 1X Tris-acetic acid-ethylenediaminotetraacetic acid, Na salt (EDTA) (TAE) buffer (40nM Tris-acetate, 2mM $Na_2EDTA.2H_2O$; pH 8.0). 10% concentration of ethidium bromide was added to the mixture. 1X DNA loading dye (Thermoscientic) was added to the DNA samples and loaded into the wells of the agarose gel, submerged in 1x TAE Buffer, and separated at 100 volts.

Cell Culture*:* Bone-marrow cells from femurs and tibias were extracted from adult mice and myeloid lineage was enriched by plating on non-adherent plastic plates in Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 50 U/ml penicillin/streptomycin (Life Technologies, Carlsbad, CA) and 50ng/mL recombinant human CSF-1 (Peprotech, Rocky Hill, NJ) for three days. After three days in this culture, the non-adherent myeloid precursors were mechanically isolated and transferred to adherent tissue culture plates, 5 x 10^6 cells per 10cm plate. These cells were cultured in DMEM with 10% FBS, 50 U/mL Penicillin/Streptomycin, 50 ng/mL CSF-1 for five days. 100ng/mL recombinant human RANKL was added at day three (Peprotech, Rocky Hill, NJ). These cells were harvested at times indicated in the figures.

Chromatin Immunoprecipitation (ChIP): BMMs from wild-type mice were plated and treated with the differentiation-cytokines as indicated above. Before harvesting, the cells were treated with 1% concentration formaldehyde at 37°C for 10min. Nuclear extracts were sonicated by a Branson 250 digital sonicator (Branson Ultrasonics, Danbury, CT) to create DNA fragments of 200-600bp. ~5 x 10^5 cell equivalents of the fragmented DNA were treated with

Protein G-agarose beads. Roughly 10% of this DNA was set aside as an input control, the rest was treated with 5µg of EOMES antibody (Abcam). This was left overnight at 4°C. The Protein G-agarose was pulled down, washed extensively, and eluted twice with 250 L of elution buffer (0.1 M $NaHCO_3$, 1% SDS). The cross-linking was reversed with 200mM NaCl and 20µg of RNase A (Sigma-Aldrich, St. Louis, MO) and left overnight. The isolated DNA was treated with Proteinase K (Roche, Basel, Switzerland) and purified with the Qiagen PCR purification kit (Invitrogen, Life Technologies, Carlsbad, CA). These samples were analyzed by qPCR on a StepOnePlus machine (Applied Biosystems, Life Technologies, Carlsbad, CA) using Taqman MasterMix (Roche, Basel, Switzerland) and Universal Probe Library Probes (Roche, Basel, Switzerland). The primer for *Nfatc1* binding sites was designed using Universal Probe Library software (Roche, Basel, Switzerland) (Table 2). The threshold was adjusted according to the input values, calculated using a variation of the ddCt method (27) and expressed in relative enrichment.

Forward	5'-TGCACTAGTTCCGCTTTCTTT-3'
Reverse	5'-CTGTCAGTATGGGGGATGCT-3'

Table 2: Primers designed for the EOMES, MITF, and PU.1 binding site located 5kbp from the *Nfatc1* promoter region

MicroCT Imaging of Mouse Skeletons: Bones of experimental and control mice were extracted from eight-day-old mice, fixed with 4% paraformaldehyde for 48hrs, and then stored in 70% ethanol until use. Radiograph images were taken using a Siemens Inveon Preclinical CT scanner (Siemens AG, Munich, Germany) with settings at 100kV, 200MA, 1 second exposure, Bin 2, and a medium-high system magnification with a resolution of 0.019mm isotropic voxels. Image reconstruction was done using Cobra software (Exxim, Pleasanton, CA) and analyzed using 3D

bone morphology analysis software (Inveon Research Workplace 3D Image Software, Siemans PreClinical, Munich, Germany). Mineral bone density was measured using a ratio of mineralized bone to total bone volume (BV/TV). Trabecular density was measured in thickness of the trabecular (Tb.Th) (mm), trabecular number (Tb.No) (mm^{-1}), and trabecular separation (Tb.Sp) (mm).

Bone Histomorphometry: Femurs from eight day old mouse femurs were fixed in 3.7% paraformaldehyde, embedded in paraffin wax, cut into 4 μm sagittal sections, and stained for hematoxylin & eosin and tartrate-resistant acid phosphatase (TRAP) using a Leukocyte Acid Phosphate kit (Sigma-Aldrich, St. Louis, MO). These slides were scanned on an Aperia ScanScope XT (Vista, CA) and quantified using Aperio ImageScope software (Vista, CA). The osteoclasts present were measured using the ratio of osteoclast-covered surface to total bone surface (Oc.S/BS) and osteoclast number per millimeter of trabecular perimeter (Oc.No./Tb. pm).

RNA Extraction, cDNA Preparation and Quantitative Real-Time PCR (qRT-PCR): Total RNA was extracted from the RANKL/CSF1 treated BMMs using TRIzol method (Invitrogen), and purified according to the manufacturer's protocol. 2μg of purified RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA) with random hexamer primers. Primers for the osteoclast-specific genes were designed using the Universal Probe Library software (Roche, Basel, Switzerland) (Table 3). qPCR was done using Taqman Master Mix (Roche, Basel, Switzerland) on a StepOnePlus machine (Applied Biosystems, Life Technologies, Carlsbad, CA). Relative expression of the target

mRNA compared to the ribosomal protein L4 internal control was calculated using a variation of the ddCt method (27).

Gene	Left	Right
Acp5	5'-CGTCTCTGCACAGATTGCAT-3'	5'-AAGCGCAAACGGTAGTAAGG-3'
Oscar	5'-TCTGCCCCCTATGTGCTATC-3'	5'-TAGTCCAAGGAGCCAGAACC-3'
Ctsk	5'-CGAAAAGAGCCTAGCGAACA-3'	5'-TGGGTAGCAGCAGAAACTTG-3'
Calcr	5'-GGTTCCTTCTCGTGAACAGGT-3'	5'-AGAACTGGAGTTGGGCTCAC-3'

Table 3: Primers used for RT-PCR analysis

Statistical Analysis: Unpaired student's t-tests were done using Microsoft Excel for all statistical analysis. Results with differences with $P < .05$ were considered significant.

IV. Results

EOMES co-binds to *Nfact1* locus with MITF and PU.1:

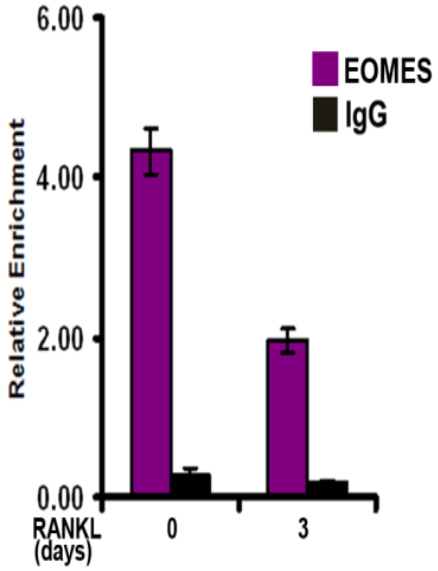

Figure 7: ChIP with qPCR analysis confirmed EOMES binding to *Nfatc1*.

Motif analysis revealed the binding sequence for EOMES at 40% of the sites shared by MITF and PU.1, suggesting that EOMES plays a role in osteoclast differentiation. Before any subsequent experiments of EOMES in osteoclast differentiation, the results shown by motif analysis needed to be confirmed.

Since it was revealed that an EOMES binding sequence is 5kbp from the promoter of *Nfatc1,* ChIP with EOMES antibodies was performed, using DNA from wild type BMMs treated with CSF-1 alone, or with RANKL for three days. To quantify the enrichment of EOMES, primers designed from the sites of MITF, PU.1 and EOMES binding sequences on *Nfatc1* were used for qRT-PCR of the DNA bound by EOMES.

The results from these experiments verified EOMES binding near the promoter of *Nfatc1* during osteoclast differentiation: the binding was present following CSF-1 treatment and persisted after addition of RANKL signaling (Figure 7). These results mimic those found through validation of MITF and PU.1 binding.

Mice with myeloid specific knockout of EOMES exhibit an osteopetrotic phenotype due to decreased osteoclast production:

An EOMES knockout mouse model was given to the Ostrowski laboratory to analyze the role of EOMES in osteoclast differentiation. Since complete *Eomes* allele deletion is embryonic lethal, we used a mouse model in which there is a conditional knockout of a "floxed" *Eomes* allele under a myeloid specific promoter (EOMES $^{\Delta Mye/\Delta Mye}$). MicroCT analysis of the femurs of EOMES WT and EOMES $^{\Delta Mye/\Delta Mye}$ mice determined the level of osteopetrosis, revealing an approximately 10% increase in mineral bone volume to total bone volume (BV/TV) due to increased trabecular number (Tb. No/mm) and thickness of the trabecular (Tb. Th.), and decreased space between trabecular bone (Tb. Sp.) in EOMES $^{\Delta Mye/\Delta Mye}$ mice (Figure 7 A,B).

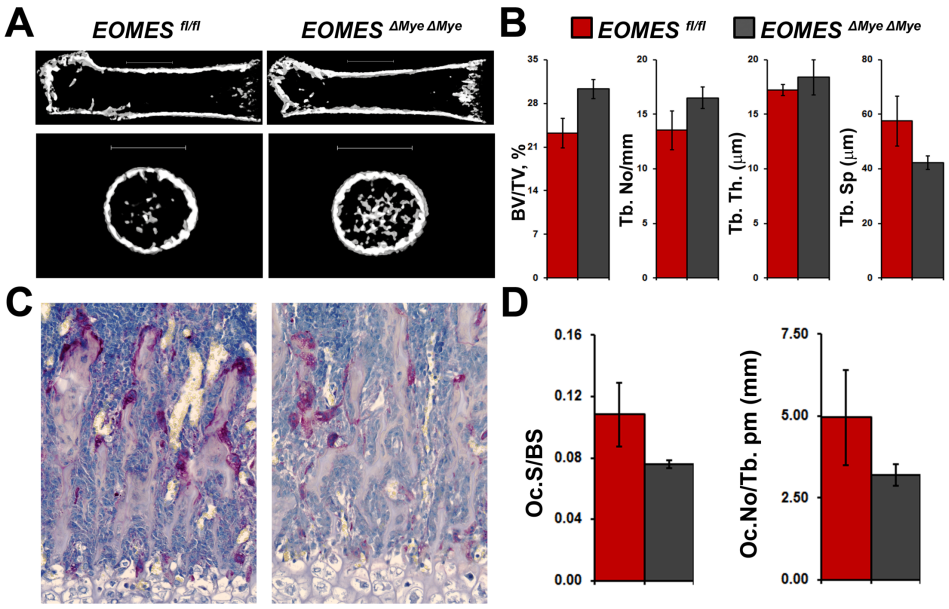

Figure 8: A. Micro CT image of EOMES cKO mice show osteopetrotic phenotype, though milder than PU.1 cKO mice. B. Quantification of bone volume to total volume, and measurements of the trabecular bone. C. TRAP staining of *A* show decreased levels of osteoclast production. D. Quantification of osteoclast surface area:total bone surface area and osteoclast number: trabecular perimeter. (Carey,H. and Hildreth, E.)

To verify whether the mild osteopetrosis observed in EOMES $^{\Delta Mye/\Delta Mye}$ is osteoclast specific, the femur sections were stained for the osteoclast marker, TRAP. TRAP staining of the femurs showed a significant decrease in active osteoclasts in EOMES $^{\Delta Mye/\Delta Mye}$ mice. (Figure

7C). There was a decrease in the ratio of osteoclast-covered surface to total bone surface (Oc.S/BS) and osteoclast number per millimeter of trabecular perimeter (Oc.No./Tb. pm) (Figure 7D).

Myeloid-specific knockout of EOMES causes a decrease in *Nfatc1* expression:

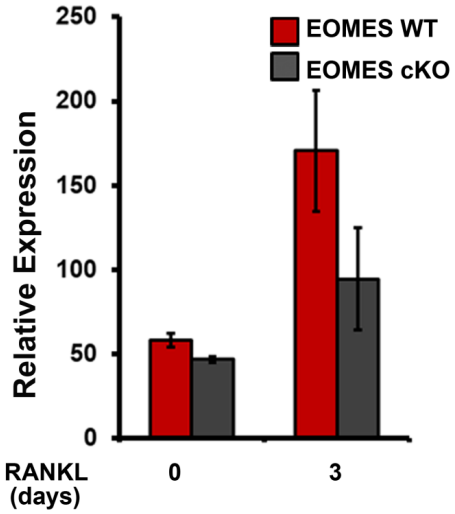

Figure 10: qPCR of EOMES $^{\Delta Mye/\Delta Mye}$ RNA showed a decrease in expression of *Nfatc1* following RANKL treatment.

NFATC1 is important member of the osteoclast transcription network, implicated in the regulation of terminal osteoclast regulation. Since EOMES was found to be associated with MITF-PU.1 bound loci of *Nfatc1* enhancers, the deregulation of the transcription factor network may be the cause of lowered osteoclast production seen in EOMES $^{\Delta Mye/\Delta Mye}$ mice. To validate this, we analyzed the effect of EOMES knockdown on *Nfatc1* expression.

Total RNA was isolated from EOMES $^{\Delta Mye/\Delta Mye}$ and EOMES WT BMMs treated with and without RANKL. qRT-PC showed a two-fold *Nfatc1* expression decrease in osteoclast from EOMES $^{\Delta Mye/\Delta Mye}$ mice following RANKL treatment (Figure 10), which contributes to a deregulated osteoclast transcription factor network. The timing of the expression regulation is consistent with PU.1 and MITF, proving that EOMES co-regulates upstream in the transcription factor network of osteoclast differentiation with MITF and PU.1.

Knockout of EOMES in the myeloid compartment results in decreased expression of osteoclast-effector genes:

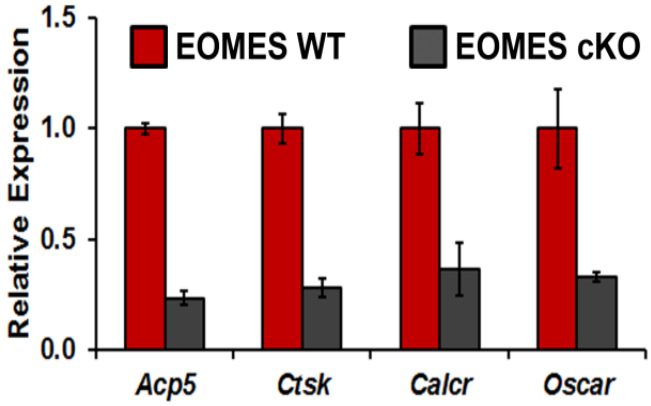

Figure 9: qPCR data depicts decrease in osteoclast effector genes in EOMES$^{\Delta Mye/\Delta Mye}$ mice.

Since the transcription factor network of osteoclast differentiation becomes deregulated following myeloid cell knockout of EOMES, it should lead to a decrease in the genes controlled by the network.

BMMs of EOMES $^{\Delta Mye/\Delta Mye}$ and EOMES WT mice were harvested and treated with CSF-1 for two days, followed by CSF-1 and RANKL for three days. The RNA from these cells was isolated and qRT-PCR found the level of expression of osteoclast effector genes *Acp5, Calcr, Ctsk,* and *Oscar.*

When compared to osteoclasts from EOMES WT mice, osteoclasts from EOMES$^{\Delta Mye/\Delta Mye}$ mice exhibited an approximately three-fold reduction in the expression of osteoclast marker genes (Figure 9). Each of these genes down regulated in cells lacking EOMES are necessary for the differentiation of myeloid cells to active osteoclasts. This explains the decrease in active osteoclast in EOMES $^{\Delta Mye/\Delta Mye}$ mice and exhibits the regulatory function EOMES serves in osteoclast differentiation.

V. Discussion and significance

The differentiation of osteoclast is unique from the differentiation processes of other cell types. Whereas differentiation to many cells types, such as muscle cells or melanocytes, requires

simple mechanisms of transcriptional regulation, differentiation to active osteoclast requires a complex transcription factor network. Knocking out individual members of this network results in osteopetrotic phenotypes in mice, showing that the function of every member of this network is vital for normal differentiation. While many of members of the network have been discovered, the complete mechanism of this network is still largely unknown. In this study, we revealed EOMES as another member of the transcription factor network of osteoclast differentiation.

Discerning the factors and mechanisms involved in osteoclast differentiation is important to finding potential targets for effective and safe treatments of bone related ailments, such as osteoporosis and rheumatoid arthritis. While there are current treatment options for bone-related diseases, many of the treatments only succeed in slowing the progression of the disease, such as reducing fractures, or work to completely abrogate the differentiation of osteoclast (24). Although decreasing osteoclast production is successful in increasing bone mass, it completely ignores the importance of bone remodeling for maintaining healthy bones. Complete knowledge of osteoclast differentiation and function could reveal therapeutic targets for tailored treatments of excessive bone degradation that would allow for modulation of osteoclast activity, rather than complete ablation of osteoclast differentiation and function.

Knocking out individual members of the transcription factor network results in varying levels of osteopetrosis. A transcription factor in which osteopetrosis is mild after its deletion/mutation may prove to be a profound target for treatments. Results from this study shows promise for EOMES as a target. PU.1 and MITF have an early, established role in osteoclast differentiation. As a complex, they increase the production of osteoclast-specific genes dramatically. These factors would be unsuitable as a drug target, due to their large role in differentiation, and the role of PU.1 in many different myeloid lineages. This study showed that

EOMES co-regulates with MITF and PU.1, but the EOMES binding site is found in only 40% of the DNA sites bound by MITF and PU.1. While it does have a regulatory role in osteoclast differentiation, it is not as significant as its co-partners. There was only a ten percent increase in bone mass in EOMES knockout mice, as compared to a twenty percent increase in PU.1 knockout mice. PU.1 knockout BMMs showed almost a complete loss of osteoclast-effector gene expression, whereas EOMES knockout BMMs exhibited a decrease in the expression of these genes, but the expression is not completely halted.

Because of the potential of EOMES, it is important to continue studies of the transcription factor in osteoclast differentiation. Co-immunoprecipitation with EOMES, MITF and PU.1 could give insight into whether the EOMES and MITF/PU.1 regulation occurs in the same complex, as well as reveal their respective domains of interaction. Additionally, ChIP-seq of EOMES would show the extent of EOMES regulation outside of co-regulation with MITF and PU.1. Not only could this expose separate functions of EOMES in osteoclast differentiation, but like the results found from MITF/PU.1 ChIP-seq, motif analysis of these peaks could expose other members of the regulatory network.

1. “What is bone?” *National Institute of Arthritis and Musculoskeletal and Skin Diseases.* National Institute of Health, May 2015.
2. “Introduction to Bone Biology: All About Our Bones.” *International Osteoporosis Foundation.* International Osteoporosis Foundation, 2015.
3. “What is Bone Health in America?” Office of the Surgeon General (US). Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville (MD): Office of the Surgeon General (US); 2004, 2.
4. “Osteopetrosis.” *Genetics Home Reference.* Genetics Home Reference, Sept 2010. Web.
5. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423(6937):337-42. doi: 10.1038/nature01658. PubMed PMID: 12748652.
6. Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-kappa B ligand (RANKL)-induced JNK activation. The Journal of biological chemistry. 2001;276(12):8836-40. doi: 10.1074/jbc.M010764200. PubMed PMID: 11121427.
7. Rheumatoid Arthritis | American College of Rheumatology | ACR." *Rheumatoid Arthritis | American College of Rheumatology | ACR*. N.p., n.d. Web. 12 Feb. 2014.
8. Sato K, Takayanagi H. Osteoclasts, rheumatoid arthritis, and osteoimmunology. Current opinion in rheumatology. 2006;18(4):419-26. doi: 10.1097/01.bor.0000231912.24740.a5. PubMed PMID: 16763464.
9. Roodman GD. Biology of osteoclast activation in cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2001;19(15):3562-71. PubMed PMID: 11481364.
10. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. Journal of cell science. 2000;113 (Pt 3):377-81. PubMed PMID: 10639325.
11. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. Bone. 2007;40(2):251-64. doi: 10.1016/j.bone.2006.09.023. PubMed PMID: 17098490.
12. Wong BR, Josien R, Lee SY, Vologodskaia M, Steinman RM, Choi Y. The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. The Journal of biological chemistry. 1998;273(43):28355-9. PubMed PMID: 9774460.
13. Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science. 1994;266(5184):443-8. PubMed PMID: 7939685.
14. Mansky KC, Sankar U, Han J, Ostrowski MC. Microphthalmia transcription factor is a target of the p38 MAPK pathway in response to receptor activator of NF-kappa B ligand signaling. The Journal of biological chemistry. 2002;277(13):11077-83. doi: 10.1074/jbc.M111696200. PubMed PMID: 11792706.
15. Luchin A, Purdom G, Murphy K, Clark MY, Angel N, Cassady AI, Hume DA, Ostrowski MC. The microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2000;15(3):451-60. doi: 10.1359/jbmr.2000.15.3.451. PubMed PMID: 10750559.
16. Motyckova G, Weilbaecher KN, Horstmann M, Rieman DJ, Fisher DZ, Fisher DE. Linking osteopetrosis and pycnodysostosis: regulation of cathepsin K expression by the microphthalmia transcription factor family. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(10):5798-803. doi: 10.1073/pnas.091479298. PubMed PMID: 11331755; PubMed Central PMCID: PMC33293.

17. So H, Rho J, Jeong D, Park R, Fisher DE, Ostrowski MC, Choi Y, Kim N. Microphthalmia transcription factor and PU.1 synergistically induce the leukocyte receptor osteoclast-associated receptor gene expression. The Journal of biological chemistry. 2003;278(26):24209-16. doi: 10.1074/jbc.M302940200. PubMed PMID: 12695521.
18. Granholm S, Lundberg P, Lerner UH. Expression of the calcitonin receptor, calcitonin receptor-like receptor, and receptor activity modifying proteins during osteoclast differentiation. Journal of cellular biochemistry. 2008;104(3):920-33. doi: 10.1002/jcb.21674. PubMed PMID: 18384073.
19. Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, Bachler MA, Amano H, Aburatani H, Ishikawa H, Wagner EF. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. The Journal of biological chemistry. 2004;279(25):26475-80. doi: 10.1074/jbc.M313973200. PubMed PMID: 15073183.
20. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. The EMBO journal. 1996;15(20):5647-58. PubMed PMID: 8896458; PubMed Central PMCID: PMC452309.
21. Moore KJ. Insight into the microphthalmia gene. Trends in genetics : TIG. 1995;11(11):442-8. PubMed PMID: 8578601.
22. Carey, et al. Failure to Target RANKL Signaling Through p38-MAPK Results in Defective Osteoclastogenesis in the Microphthalmia Cloudy-Eyed Mutatn. J Cell Physiol. 2016 Mar;231(3):630-40. doi: 10.1002/jcp.25108. PubMed PMID: 26218069.
23. Luchin A, Suchting S, Merson T, Rosol TJ, Hume DA, Cassady AI, Ostrowski MC. Genetic and physical interactions between Microphthalmia transcription factor and PU.1 are necessary for osteoclast gene expression and differentiation. The Journal of biological chemistry. 2001;276(39):36703-10. doi: 10.1074/jbc.M106418200. PubMed PMID: 11481336.
24. Sharma SM, Bronisz A, Hu R, Patel K, Mansky KC, Sif S, Ostrowski MC. MITF and PU.1 recruit p38 MAPK and NFATc1 to target genes during osteoclast differentiation. The Journal of biological chemistry. 2007;282(21):15921-9. doi: 10.1074/jbc.M609723200. PubMed PMID: 17403683.
25. Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, Banica M, DiCioccio CB, Gross DA, Mao CA, Shen H, Cereb N, Yang SY, Lindsten T, Rossant J, Hunter CA, Reiner SL. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science. 2003;302(5647):1041-3. doi: 10.1126/science.1090148. PubMed PMID: 14605368.
26. Zhu Y, Ju S, Chen E, Dai S, Li C, Morel P, Liu L, Zhang X, Lu B. T-bet and eomesodermin are required for T cell-mediated antitumor immune responses. Journal of immunology. 2010;185(6):3174-83. doi: 10.4049/jimmunol.1000749. PubMed PMID: 20713880.
27. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.